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ORIGINAL PAPER

A wheat genomic DNA fragment reduces pollen transmission of maize transgenes by reducing pollen viability

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Abstract A genomic DNA fragment from wheat carrying the *Glu-1Dx5* gene has been shown to exhibit reduced pollen transmission in transgenic maize. To localize the region of the DNA fragment responsible for this reduced pollen transmission, we produced transgenic maize plants in which the wheat genomic DNA proximal to the 1Dx5 coding sequence was replaced with the maize 27 kDa γ-zein promoter. Like the wheat promoter-driven *Glu-1Dx5* transgene, this zein promoter-driven transgene functioned to produce 1Dx5 in maize endosperm. However, with the zein promoter-driven transgene, pollen transmission of the transgene loci was normal in most self- and cross-pollinations. We concluded that the wheat genomic DNA proximal to the wheat 1Dx5 coding sequence was required for reduced pollen transmission of the transgene in maize. In two of four transformation events of the wheat promoter-driven construct examined, pollen exhibited two morphological classes. In one class, pollen was normal in morphology and displayed average viability, and in the second, pollen was reduced in size and did not germinate on artificial media. DNA from the transgene was detectable in mature pollen from plants with reduced pollen transmission of transgene loci. To explain these observations, we hypothesize that elements within the transgene construct interfere with pollen development. We demonstrated that the wheat genomic DNA fragment can be used to control pollen transmission of an herbicide resistance transgene genetically linked to it. The wheat genomic DNA fragment may contain elements that are useful for controlling pollen transmission of transgene loci in commercial maize grain and seed production.

Keywords Glu1-Dx5 · Pollen viability · Transgene control

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Introduction

Biotechnology offers great potential for crop improvement by enabling foreign gene products to be produced in crop plants. While this technology can improve the quality, quantity, and value of crops, it also has created a need to control the inadvertent genetic transmission of transgenes through pollen. Dispersal of maize

(*Zea mays* L.) pollen has been modeled (Aylor et al. 2003) and the extent of cross-fertilization of maize by a given pollen source has been shown to be dependent on distance from the source, wind strength and direction, and flower synchrony (Aylor et al. 2003; Halsey et al. 2005; Ma et al. 2004).

A number of methods for controlling transgene dispersal by pollen have been proposed and reviewed (Daniell 2002). A strategy for controlling pollen transmission using the *Teosinte crossing barrier 1* (*Tcb1*) gene also has been proposed (Evans and Kermicle 2001). Efficient containment of pollen by detasseling plants which carry transgenes also has been demonstrated (Stevens et al. 2004). It seems likely that different methods or combinations of methods will be required to suit different maize production systems. A desirable system would not require planting two varieties to produce grain and would not require the use of a specific genetic background. Maternal inheritance would meet these requirements and can be conferred on a transgene by organellar transformation (Maliga 2004). Unfortunately, this technology has not been developed for maize, and will not work for traits that require expression from the nuclear genome.

The *Glu-1Dx5* gene in wheat (*Triticum aestivum*) encodes the high molecular weight (HMW) glutenin subunit 1Dx5, a prolamin seed storage protein with no known enzymatic activity. This gene has been isolated and its sequence has been determined (Anderson et al. 1989). It functions to produce the 1Dx5 high molecular weight glutenin subunit specifically in wheat endosperm. Sangtong et al. (2002) studied the inheritance of the *Glu-1Dx5* gene in the maize nuclear genome in four transformation events for three generations. Pollen transmission of the transgene was not observed in two of the events, and was observed in one generation at a frequency of less than 10% in the other two events. It may be possible to utilize an element similar to this transgene to confer maternal inheritance on other transgenes for the purpose of controlling dispersal of these transgenes through pollen. If another transgene is co-transformed with a transgene that prevents pollen transmission so that the two transgenes are physically linked, it is likely that they would be genetically linked as well. This should result in both transgenes exhibiting reduced transmission through pollen.

The specific objectives of this study were: (1) to determine if sequences 5′ of the 1Dx5 coding sequence in the P2P31 construct are necessary to give poor pollen transmission of the transgene locus; (2) to develop an hypothesis about the mechanism of pollen control; and (3) to establish the feasibility of using a transgene that confers reduced pollen transmission to control pollen transmission of a transgene that is genetically linked to it.

Materials and methods

Development of transgenic plants expressing 1Dx5 using the maize gamma zein promoter

In order to identify the region of the wheat genomic DNA fragment contained within the construct designated P2P31 that exhibited the unusual pollen transmission reported previously (Sangtong et al. 2002), a transgene construct designated P2P46 was developed in which the 5′ flanking region of the wheat genomic fragment proximal to the sequence coding for 1Dx5 was replaced with the maize γ-zein promoter (Fig. 1, GenBank Accession DQ907161). Thus, the P2P46 construct encodes an exact copy of the 1Dx5 protein produced by the P2P31 construct, however, it contains none of the wheat sequences 5′ of the translation initiation codon. The P2P46 construct was used to transform maize embryonic

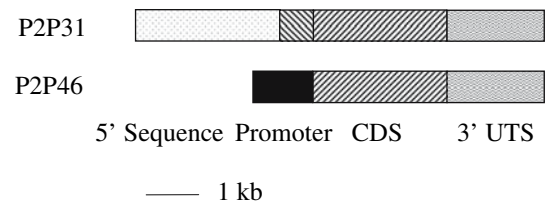

Fig. 1 P2P46 and P2P31 *Glu-1Dx5* construct design. P2P46 construct lacked wheat 5′ upstream region (stippled) and promoter sequence (downward slanting cross hatches). The promoter sequence in P2P46 construct was from γ-zein (black). The coding sequence (CDS, upward slanting cross hatches) and 3′ untranslated region (UTS, wavy lines) were unchanged

callus of the genotype HiII using microprojectile bombardment (Frame et al. 2000). Herbicide-resistant callus lines carrying the transgene were identified by PCR and were regenerated to plants. These plants were crossed to the non-transgenic inbred line B73 to give F_1 kernels. All P2P46 events used in this study were derived from these F_1 kernels, which were self pollinated or crossed to non-transgenic plants in subsequent generations.

Detection of transgene product

In each generation, kernels were screened for the presence of 1Dx5 in the endosperm using an antibody specific to 1Dx5 in an ELISA, western blot, or tissue print immuno assay. Endosperm tissue was sampled so as to not damage the embryo, and transgene positive seeds were either planted for the next generation or used for experiments.

For ELISA assays, protein was extracted from ground endosperm in extraction buffer containing 70% ethanol, 61 mM sodium acetate, 5% β-mercaptoethanol (β-ME) at a ratio of 1 mg endosperm tissue to 10 μl extraction buffer. This extract was diluted with an equal volume of the extraction buffer and bound to an ELISA plate at 37°C overnight. The ELISA assay was carried out according to Harlow and Lane (1988) using rabbit polyclonal antibody raised to synthetic 1Dx5 peptides, and a secondary antibody specific to rabbit IgG conjugated to alkaline phosphatase (AP). AP activity was quantified using a colorimetric assay in a microtiter plate spectrophotometer.

Western blots were carried out following denaturing SDS-PAGE (Laemmli 1970) using a Bio-Rad Mini Trans-Blot® cell (Hercules, CA) to transfer proteins to a nylon-backed nitrocellulose membrane or a PVDF membrane according to the manufacturer's directions. Immuno-detection was carried out using a primary antibody consisting of polyclonal antisera raised in rabbit against peptides from the wheat 1Dx5 subunit. Detection was carried out using an alkaline phosphatase conjugated secondary antibody and a colorimetric detection kit (Bio-Rad, Hercules, CA).

The tissue print immuno assay was carried out by first sanding the top of the kernel to a flat surface with sandpaper to expose the endosperm. Seven to 10 μl of extraction buffer (50 mM Tris–Cl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 1% β-ME) were pipetted onto the exposed endosperm. After 2 min, the kernel was pressed onto a sheet of nylon-backed nitrocellulose membrane. This membrane was processed in the same way as the western blots.

Plant material

In 2004, plants from seven transformation events containing 1Dx5-expressing constructs including four with P2P31 (Sangtong et al. 2002), three with P2P46, and B73 inbred were grown. In 2005, plants containing only P2P31 and B73 were grown.

Kernels from P2P46 and P2P31 events positive for the transgene and from ears that were segregating for the presence of 1Dx5 were selected and grown in the same field as the inbred line B73 for comparison of inheritance patterns and for pollen studies.

Individual plants were tagged and controlled self pollinations were carried out on transgenic plants and on non-transgenic B73. To confirm the genotype of the transgenic plants and to assess pollen transfer of the transgene, controlled pollinations also were carried out with transgenic plants as the pollen source and non-transgenic B73 plants as the maternal parent. Ears were harvested about 50 days after pollination, dried to between 12% and 15% moisture and shelled with the grain from each ear kept separate from that of the other ears.

Transgene transmission analysis

The inheritance patterns of the selectable *bar* gene marker and the 1Dx5 transgene in the self-pollinated, transgenic ears and B73 x transgenic ears described above were assessed and compared by PCR. One hundred fifty randomly selected kernels from representative ears were planted in the greenhouse in flats containing a soil-peat-sand mix. Genomic DNA was extracted by the CTAB method of Saghai-Maroof, et al. (1984), with some modifications, from freeze-dried, pulverized, two-week-old leaf tissue. The DNA pellet was resuspended in water containing RNase. All

PCR reactions contained 1 μl isolated DNA (~50 ng), 200 μM each dNTP (Invitrogen, Carlsbad, CA), 10 μl 5x GoTaq® PCR buffer and 1.25 U GoTaq® DNA polymerase (Promega, Madison, WI) and 20 pmol each of forward and reverse primers in a 50 μl volume. Primers for amplification of the *bar* gene were designed from the sequence published by Vickers et al. (1996): forward 5′-CAGGAACCGCAGGAGTGGA-3′, and reverse 5′-CCAGAAACCCACGTCATGCC-3′. For amplification of the 1Dx5 gene, primer sequences unique to the 1Dx5 gene were as follows: forward 5′-TAAGCGGTTAGTCCTCTTTGTG-3′ and reverse 5′-TGCTGACCTTGTTGCCCTTG-3′. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR conditions were 2 min initial denaturation period at 94°C followed by 31 cycles of 30 s at 56°C (1Dx5 gene amplification) or 60°C (*bar* gene amplification), 2 min at 72°C, and 30 s at 94°C, with a final extension period of 2 min at 72°C on a MJ Research DNA Engine PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, CA). Products were resolved on a 1% agarose gel (Amresco, Solon, OH).

Glu-1Dx5 phenotypic transmission analysis

Segregation ratios were determined on cross- and self-pollinated ears of P2P31 plants and P2P46 plants by analyzing kernels using the tissue print immuno assay method to provide a qualitative assessment of the presence or absence of the transgene product. Segregation ratios were assigned using a min-max test to distinguish between 1:1 and 3:1 segregation ratios (Tables 1, 2).

Bar gene phenotypic transmission analysis

Plants were sprayed after tissue collection at the four-leaf stage with a 2.5% (v/v) solution of Liberty 200 SL (active ingredient glufosinate ammonium, 18.19%, Bayer Cropscience, Kansas City, MO) and 0.1% (v/v) Tween® 20 (Sigma-Aldrich Co., St. Louis, MO) (Brettschneider et al. 1997). Plants were evaluated for herbicide resistance at 7 days and 10 days after herbicide application (Table 3).

Table 1 Transgene segregation in F_2 kernels of P2P46

Event	Number positive	Number negative	Ratio	Segregation ratio accepted
P2P46-07	23	7	0.766[a]	3:1
P2P46-38	22	8	0.733	3:1
P2P46-18	27	3	0.900	3:1
P2P46-42	25	5	0.833	3:1

Probabilities for $n = 30$ of making a wrong decision between the test cross ratios 1:1 and 3:1 are 1:1 = 0.1002 and 3:1 = 0.0507

[a] Calculated dividing line ratio for accepting 1:1 vs. 3:1 ratio is 0.6309

Characterization of pollen

To test for the presence of the transgene in both mature pollen and anthers, DNA was extracted and purified from both separately for PCR analyses. Mature anthers and pollen were collected fresh at mid-morning, ground separately to powder in liquid nitrogen and extracted in ice cold extraction buffer (200 mM Tris–HCl, pH 7.5, 250 mM sodium chloride, 25 mM EDTA, 0.5% SDS) followed with an equal volume of equilibrated phenol. The samples were centrifuged for 10 min at 12,000 × *g*, the aqueous phase transferred to new tubes and mixed with one-half volume chloroform. The samples were centrifuged as before, the aqueous phase transferred to new tubes and mixed with an equal volume of ice cold isopropanol. After incubation at room temperature, the samples were centrifuged, the isopropanol was aspirated off and the tubes were inverted on a paper towel. The pellets were allowed to air dry and then were washed in 70% ethanol and again air dried. Pellets were resuspended in sterile, distilled, deionized water and stored at 4°C until PCR analysis.

PCR was carried out in 20 μl volume with 5 μl of the DNA solution, 1.5 μl primers specific to the promoter of glutenin HMW subunit gene *Glu-1Dx5* (SWDx5F: 5′-GGATTCGTGTTGCTGGAAAT-3′ and SWDx5R: 5′TGCCAACACAAAAGAAGCTG-3′), 3 μl water, and 10 μl SIGMA® JumpStart™ ReadyMix™ REDTaq™ DNA Polymerase (St. Louis, MO) at 94°C for 5 min, then 94°C for 30 s, 60°C for 1 min, 72°C for 90 s, for 35 cycles, followed by incubation at 72°C

Table 2 Segregation of *Glu-1Dx5* in self- and cross-pollinated transgenic lines based on immuno-blot assay for 1Dx5 protein

Self pollination					Cross pollination					Used in Figs.
Event	Number positive	Number negative	Ratio	Segregation ratio accepted [a]	Cross	Number positive	Number negative	Ratio	Segregation ratio accepted	
P2P31-097	16	24	0.400[a]	1:1	B73 × P2P31-097	0	40	0.000	n.t.	3c, 4c
P2P31-144	21	19	0.525	1:1	B73 × P2P31-144	1	39	0.025	n.t.	3d, 4d
P2P31-144	21	19	0.525	1:1	B73 × P2P31-144	12	28	0.300	n.t.	
P2P31-182	20	20	0.500	1:1	B73 × P2P31-182	3	37	0.075	n.t.	
P2P31-182	20	20	0.500	1:1	B73 × P2P31-182	1	39	0.025	n.t.	3e, 4e
P2P31-182	20	20	0.500	1:1	B73 × P2P31-182	3	37	0.075	n.t.	
P2P31-190	17	23	0.425	1:1	B73 × P2P31-190	0	40	0.000	n.t.	3f, 4f
P2P31-190	23	17	0.575	1:1	B73 × P2P31-190	0	40	0.000	n.t.	
P2P31-190	20	20	0.500	1:1	B73 × P2P31-190	0	40	0.000	n.t.	
P2P31-190	20	20	0.500	1:1	B73 × P2P31-190	0	40	0.000	n.t.	
P2P46-7	34	6	0.850	3:1	B73 × P2P46-7	23	17	0.575	1:1	3b, 4b
P2P46-7	21	19	0.525	1:1	B73 × P2P46-7	23	17	0.575	1:1	
P2P46-7	34	6	0.850	3:1	B73 × P2P46-7	16	24	0.405	1:1	

Probabilities for $n = 40$ of making a wrong decision between the test cross ratios 1:1 and 3:1 are 1:1 = 0.0403 and 3:1 = 0.0544

[a] Ratios above 0.350 were tested for their fit to a 1:1 or 3:1 ratio. The dividing line between these ratios is 0.631. Ratios below 0.350 were not tested (n.t.)

Table 3 Pollen germination determined on basis of pollen tube extension 1 mm or greater after 30 min

Genotype	Number of pollen grains observed	Germ (%)	SD
P2P31-097	233	41	3.15
P2P31-144	163	46	6.44
P2P31-182	113	44	7.42
P2P31-190	146	71	9.85
P2P46	54	72	7.27
B73	119	89	4.05

Percent germination is average of three replicates from each of two anthers from the P2P31 events and B73 and is the average of two replicates from each of two anthers from P2P46

for 10 min. A separate PCR was carried out with primers specific to the *bar* gene (BarF: 5′-CATCGAGACAAGCACGGTCAACTTC-3′ and BarR: 5′-TCTTGAAGCCCTGTTGCCTC-CAG-3′). In both cases, PCR products were separated on 1% agarose gel and visualized by UV fluorescence of the ethidium bromide-stained DNA.

For examining pollen morphology and viability, pollen germination media (PGM) and plates were prepared according to Schreiber and Dresselhaus (2003) with slight modifications as follows. Under aseptic conditions, equal volumes of 2× PGM and 0.6% Noble agar (Sigma, St. Louis, MO) were combined and 3 ml added to small (60 ×15 mm^2) sterile plastic Petri plates. Just prior to application of pollen, the agar/media surface was wetted with additional 2× PGM and then excess was removed.

Pollen was collected from the four events from P2P31, three events from P2P46, and also from the B73 inbred grown in the field in 2004 and from P2P31 and B73 plants grown in 2005. Tassel branches with mature, pollen-containing anthers were collected from at least two different plants from each line daily over a five to seven day period in late morning after pollen shed was initiated. They were wrapped in glassine paper and immediately transported to the lab. The tassel branches were inspected under a dissecting microscope and anthers were selected whose dehiscence pores were open but still contained pollen. Those anthers were removed with forceps individually and tapped lightly over a pollen germination media surface to evenly disseminate the pollen.

Pollen grains were photographed using a Leitz Laborlux 12 light microscope and Wild MPS 11 camera system. Images were digitized and submitted to the ISU Image Analysis Center. Pollen grain measurements were obtained by comparison to a stage micrometer scale. Measurements were made of each grain along their major and minor axes; a measure of area also was calculated for each pollen grain (Figs. 3, 4).

Pollen viability was assessed by testing pollen tube germination. Pollen grains from individual anthers were distributed over one-half of the plate surface. After 30 min at room temperature, images were captured using transmitted bright-field illumination with an Olympus SZH10 research stereo microscope (Olympus America, Inc., Melville, NY) and HRc Axiocam digital camera (Carl Zeiss, Thornwood, NY) at the Bessey Microscopy Facility, ISU. Three non-overlapping images per anther were taken. Representative images are presented in Fig. 5. Pollen germination was determined as the percentage of grains with germination tubes equal in length to the grain width after 30 min incubation.

Microspores were characterized as follows. Preemergent tassels were harvested from greenhouse-grown plants, fixed in Farmer's solution (3 parts 95% ethanol: 1 part propionic acid) and stored at –20°C. Spikelets from the midtassel region were selected and single anthers removed from the upper floret. Microspores were stained with propiocarmine solution, pressed under a coverslip and heated briefly. Digital images were captured with a Nikon Labophot microscope and Spot Insight camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Results

Creation of transgenic plants containing a modified *Glu-1Dx5* transgene

In order to understand the mechanism of the abnormal transmission observed in prior work (Sangtong et al. 2002), we sought to identify the region within the P2P31 construct conferring

abnormal pollen transmission of the locus containing this construct. We designed a second construct in which we replaced the 5′ flanking region of the wheat genomic fragment up to the sequence coding for 1Dx5 with the maize γ-zein promoter (Fig. 1). The γ-zein promoter was chosen because, like the wheat 1Dx5 promoter, it is a strong promoter that is also endosperm specific. This construct was designed to produce the same protein product with the same tissue specificity as P2P31. The construct containing the maize γ-zein promoter was designated P2P46.

Transgenic plants containing P2P46 were developed using the same methods that were used to produce the P2P31 plants (Sangtong et al. 2002). One protein band, at approximately 99–101 kDa, could be detected on western blots of P2P46 and P2P31 kernels (Fig. 2). Apparently identical bands were present in kernels transformed with the P2P31 and P2P46 constructs, consistent with the notion that these constructs encode identical proteins. Multiple bands detected in the wheat L-188 sample may indicate presence of degradation products or cross reaction with other glutenin subunits. The apparent molecular mass of the immunoreactive band in the transgenic kernels was slightly different than the 118 kDa reported earlier by Sangtong et al. (2002). We attributed this difference to differences in the gel systems and antibodies used in these studies. We used the presence of this immunoreactive protein, presumably the product of the P2P46 transgene, to identify transgenic kernels, because this protein was detected by an antibody designed to detect the transgene product and was not present in non-transgenic maize endosperm.

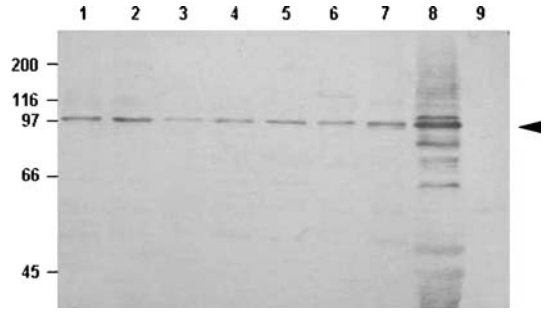

Fig. 2 Western blot of endosperm extracts from P2P31 and P2P46 events, wheat, and non-transgenic B73. Lane (1) P2P31-97; (2) P2P31-144; (3) P2P31-182; (4) P2P31-190; (5) P2P46-07; (6) P2P46-17; (7) P2P46-18; (8) Wheat L-188; (9) B73. Numbers to left indicate mass in kDa and positions of protein molecular mass standards. Arrow indicates the position of 1Dx5 subunit at approximately 101 kDa

Plants from 30 independent transformation events for P2P46 were regenerated. Western blot analysis was used to screen five kernels from each ear. Twelve events produced at least one kernel that was positive for the 1Dx5 subunit. Of these twelve events, the majority of the pollinations were made with the transgenic plant serving as the female parent. Of the twelve events with positive kernels, 11 were tested for female transmission and all of these exhibited at least one positive kernel in five tested. Knowing that Glu1-Dx5 is not transmitted efficiently through pollen in transgenic maize (Sangtong et al. 2002), we made crosses with the transgenic plant as male when possible as well. Of the 12 events with positive kernels, seven were tested for male transmission and six of these exhibited at least one positive kernel in five tested. The one plant that failed to show male transmission was propagated as a female and tested for male transmission in subsequent generations. In these studies, the progeny of the plant that did not show male transmission initially have shown normal male transmission through several generations. This was in contrast to the lack of male transmission observed in the P2P31 F_1 plants (Sangtong et al. 2002).

P2P46 inheritance analysis

We examined the inheritance of the P2P46 transgene loci in F_2 kernels derived from self pollination of P2P46 F_1 plants from four of the twelve events that produced kernels containing 1Dx5. Segregation of the transgene was monitored by immuno tissue dot blot screening for the presence of the 1Dx5 protein in endosperm of 30 kernels from each of four F_2 ears representing the four events. In all four events, the segregation ratios were not significantly different from the 3:1 ratio that would be expected for a single dominant gene with normal Mendelian inheritance, but were significantly different from the 1:1

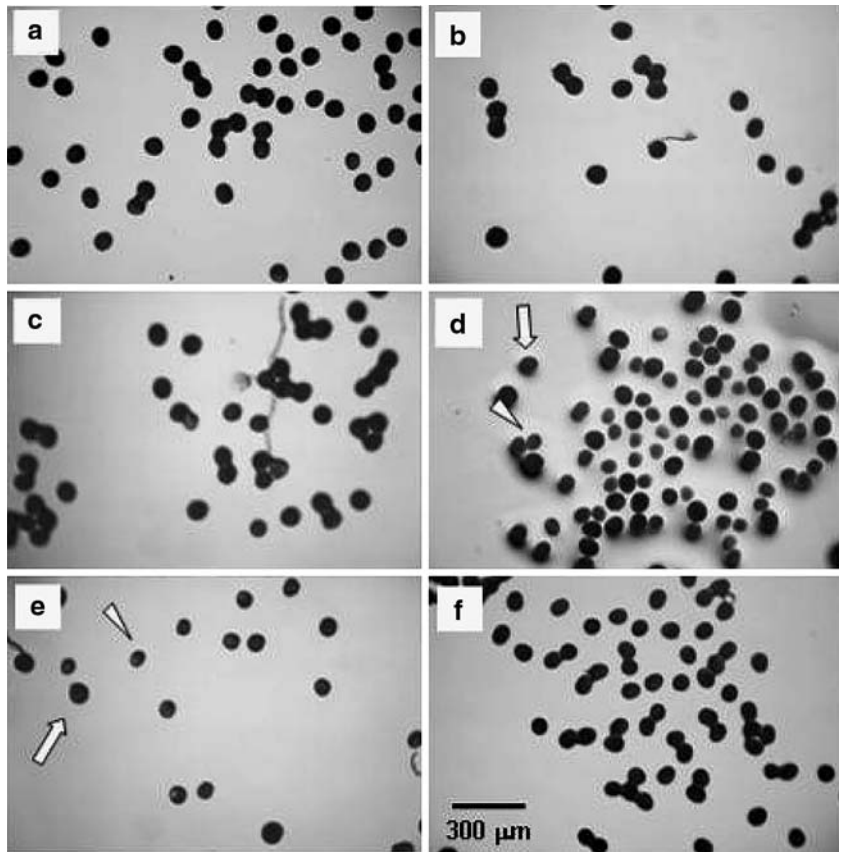

Fig. 3 Mature pollen grains from single anthers representative of heterozygous P2P31 and P2P46 field-grown plants. B73 inbred pollen also is shown. The triangles point to abnormal pollen grains, and the arrows point to normal pollen grains. (**a**) B73; (**b**) P2P46-07; (**c**) P2P31-97; (**d**) P2P31-144; (**e**) P2P31-182; (**f**) P2P31-190

segregation ratio that would be expected from a single transgene exhibiting maternal inheritance (Table 1). We therefore concluded that, unlike the plants containing P2P31, the plants containing P2P46 exhibited normal pollen transmission. Because the only difference between the P2P31 and P2P46 constructs was that the 5′ end of the P2P31 construct had been replaced by the γ-zein promoter in P2P46, we concluded sequences 5′ of the 1Dx5 coding sequence in the P2P31 construct are necessary to give poor pollen transmission of the transgene locus.

Characterization of P2P31 pollen

The second objective of this study was to develop an hypothesis to explain the reduced pollen transmission of the P2P31 construct. To do this, we characterized the morphology and viability of pollen of plants containing the P2P31 construct using P2P46 pollen as a normal pollen transmission control. P2P31 and P2P46 plants heterozygous at the transgene locus were grown side by side in the same nursery. Coincident to examining pollen from these plants, transgene inheritance and zygosity were verified by crossing these plants as males to non-transgenic B73 and by self pollination and screening for presence of 1Dx5 by immuno tissue blot assay (Table 2). Our findings related to the P2P31 construct were generally consistent with the observations of reduced male transmission in the four P2P31 events reported by Sangtong et al. (2002): (1) 1Dx5-expressing plants when self pollinated produced progeny that segregated for transgene expression at a 1:1 ratio, consistent with no pollen transmission, rather than the 3:1 ratio expected from normal pollen transmission; and (2) when the same plants were used as the male parent in crosses with non-transgenic B73, *Glu-1Dx5* expression was observed to a limited degree in the progeny of two events (144 and 182), and not at all in the other two events (97 and 190).

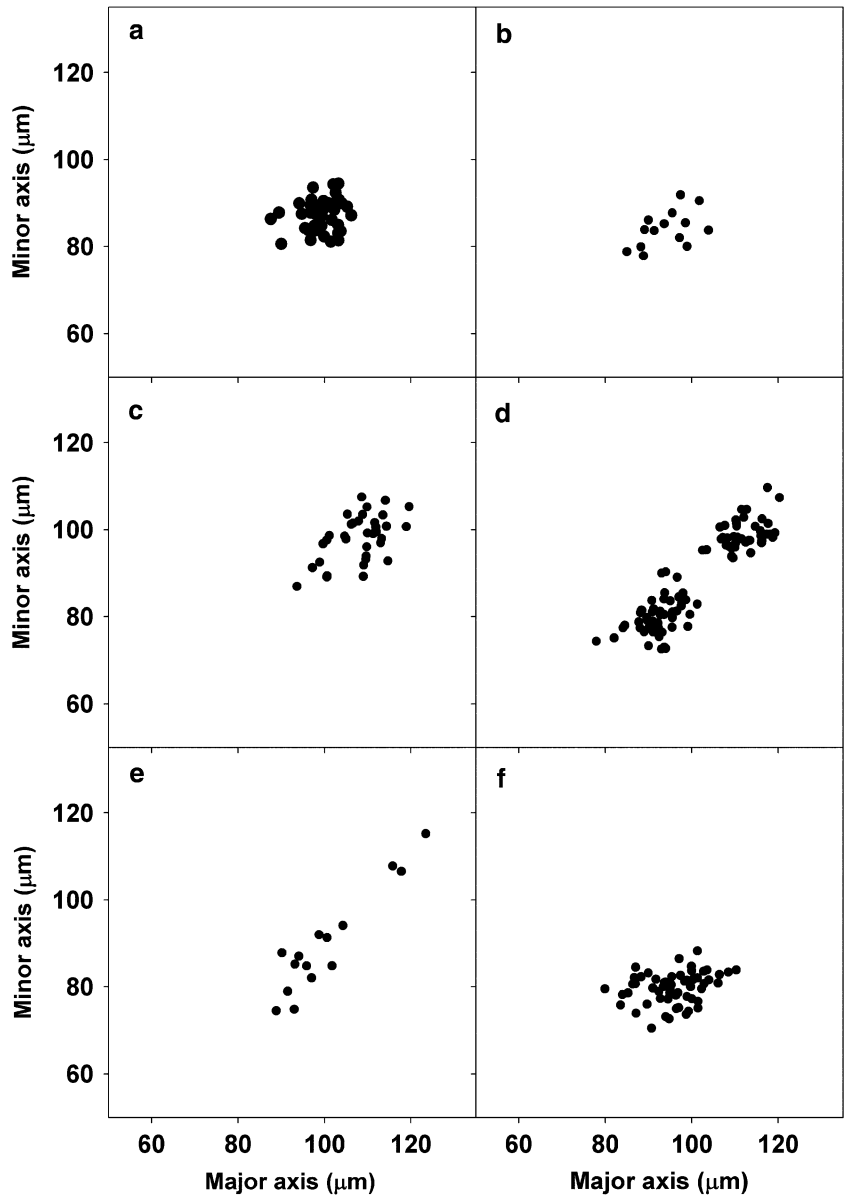

Fig. 4 Plots of the major versus minor axes in micron units of pollen grains shown in Fig. 3 reveal presence of a bimodal population of pollen grains in P2P31-144 and P2P31-182, and a non-bimodal population of pollen grains in all other events as well as the B73 inbred. (**a**) B73; (**b**) P2P46-07; (**c**) P2P31-97; (**d**) P2P31-144; (**e**) P2P31-182; (**f**) P2P31-190

Pollen morphology was determined by microscopic examination of mature pollen. Representative pollen grains taken from single anthers are presented in Fig. 3. Pollen grains from the B73 inbred, all P2P46 events, and P2P31 events 97 and 190, were visually uniform in size and shape. Some difference in average pollen grain size among anthers from a single event may reflect a moisture content effect due plant moisture status at time of harvest. By contrast, a marked difference in uniformity of pollen grains in P2P31 events 144 and 182 was clearly evident from the images and from plots of the minor versus major axes for pollen from those events (Figs. 3, 4). Pollen collected from individual anthers from the P2P31-144 event formed a distinctly bimodal population, that is, the number of pollen grains was approximately evenly divided into two groups with respect to pollen size and shape. Pollen from the other P2P31 events displayed a continuum in size along the axis from small to large. Pollen from all four of

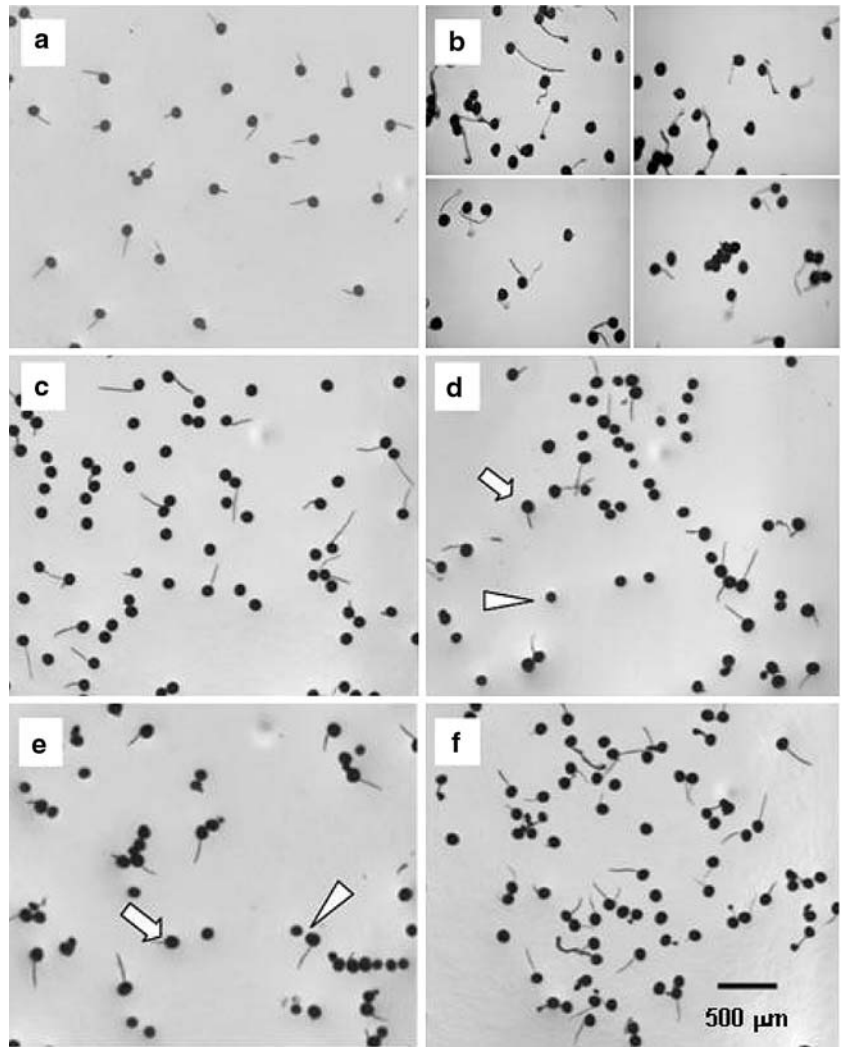

Fig. 5 Germination of pollen grains from single anthers representative of heterozygous P2P31 and P2P46 field-grown plants. The triangles point to ungerminated pollen grains and the arrows point to germinated grains. (**a**) B73; (**b**) P2P46 (four panels); (**c**) P2P31-97; (**d**) P2P31-144; (**e**) P2P31-182; (**f**) P2P31-190

the P2P31 events examined had a greater range in major axis length than any of the normal transmission controls, however the severity of the pollen morphology differences do not correlate well with the degree of pollen transmission presented in Table 2. It may be that the severity of these pollen morphology differences is event specific, although all of the P2P31 events exhibited some degree of unusual pollen morphology. This data is consistent with the hypothesis that the P2P31 construct caused a change in pollen morphology by an unknown mechanism.

One possible explanation for the unusual inheritance of the P2P31 transgene is that this transgene interferes with pollen development so that non-viable transgenic pollen is shed and non-transgenic pollen develops normally and is shed and viable. Segregation ratios indicate plants producing non-uniform pollen were heterozygous at the transgene locus. Thus, half of the pollen grains would be expected to be transgenic and the other half would not be. The morphological differences observed in the pollen could reflect differences between transgenic and non-transgenic pollen. The bimodal distribution seen in P2P31-144 plants would be an extreme expression of this morphological difference, while the elongated pattern seen in the other events in Fig. 4 would be a weaker expression of these morphological differences.

A second possibility is that the transgene interferes with pollen development, but to the

extent that transgenic pollen was not shed. In this case, the morphological differences we observed would be due to a sporophytic effect of the transgene on non-transgenic pollen development. To distinguish between the possibilities that transgenic pollen grains were not shed and transgenic pollen grains were shed but not viable, we compared the viability of pollen produced by P2P31 plants to the viability of pollen produced by B73 plants. If pollen carrying the transgene was not shed, then pollen that was shed from transgenic plants could have a normal germination frequency and the transgene still would not be transmitted through the pollen. If the pollen carrying the transgene was shed but was not viable, then we would expect pollen from transgenic plants to have a reduced percentage of viable pollen grains. Germination percentages ranged below 50% among the transgenic events with the exception of P2P31-190, which had 71% germination. B73 pollen had 89% germination (Table 3). With the possible exception of P2P31-190, these data are consistent with the hypothesis that the transgenic pollen was shed, but not viable. In the case of the pollen characterized by a bimodal population, most of the larger sized pollen germinated whereas the smaller grains did not (Fig. 5).

Thus, our data were consistent with the hypothesis that P2P31 plants have reduced pollen transmission of transgene loci due to reduced pollen viability, with transgenic pollen having lower germination rates and exhibiting varying degrees of altered morphology. At this point, we cannot yet rule out the possibility transgenic pollen aborted prior to maturity so that only non-transgenic pollen was shed. If only non-transgenic pollen was shed, the P2P31 transgene should not be present in DNA extracted from mature pollen grains. We tested this by extracting DNA from mature pollen grains from P2P31-190 plants and using PCR to amplify the transgene. A PCR product was detectable in pollen from plants identified as transgenic with the immuno tissue blot test and not present in non-transgenic sibling plants or B73 plants (data not shown). While we took extreme care to eliminate contamination of the pollen by maternal tissue, we cannot strictly rule out that this contamination is the source of the PCR product that we observed. The most likely explanation of this data, however, is that the transgene locus is present in the pollen. If the transgene DNA is present in pollen, then transgenic pollen is shed rather than aborted in development and not shed. This observation, together with the observation that non-transgenic pollen has about twice the germination rate of pollen from transgenic plants, is consistent with a model in which transgenic plants shed transgenic and non-transgenic pollen in a 1:1 ratio, and the transgenic pollen is nonviable and has varying degrees of morphological differences, while non-transgenic pollen has normal viability and morphology.

In order to establish the stage at which pollen development was perturbed, we examined binucleate microspores from P2P31 transgenic plants. This examination revealed that binucleate microspores from P2P31 events 182 and 190 appeared both morphologically uniform and cytologically similar (Fig. 6) and similar to wild-type pollen (Chang and Neuffer 1989). In contrast, pollen from event 182 at maturity displayed two morphologic classes (Fig. 3). This suggested that the two classes of mature pollen observed for event 182 were differentiated after the first mitotic stage, late in pollen development.

Pollen transmission of a linked transgene

The maternal inheritance of transgene loci conferred by the P2P31 construct could be a valuable tool for controlling pollen transmission of transgenes if the transgene of interest co-segregates with 1Dx5 because of genetic linkage. To establish the feasibility of doing this, we examined pollen transmission of the *bar* transgene which was co-bombarded with the *Glu1-Dx5* gene in the P2P31 plants. We made crosses of a transgenic male plant to a non-transgenic female plant, using each of the events containing the P2P31 construct. We tested about 150 kernels from each of these crosses by application of an herbicide containing glufosinate ammonium (Table 4). Plants carrying the *bar* gene linked to P2P46 were used as a control for normal pollen transmission, and non-transgenic B73 was used to verify that the herbicide treatment was effective. In this

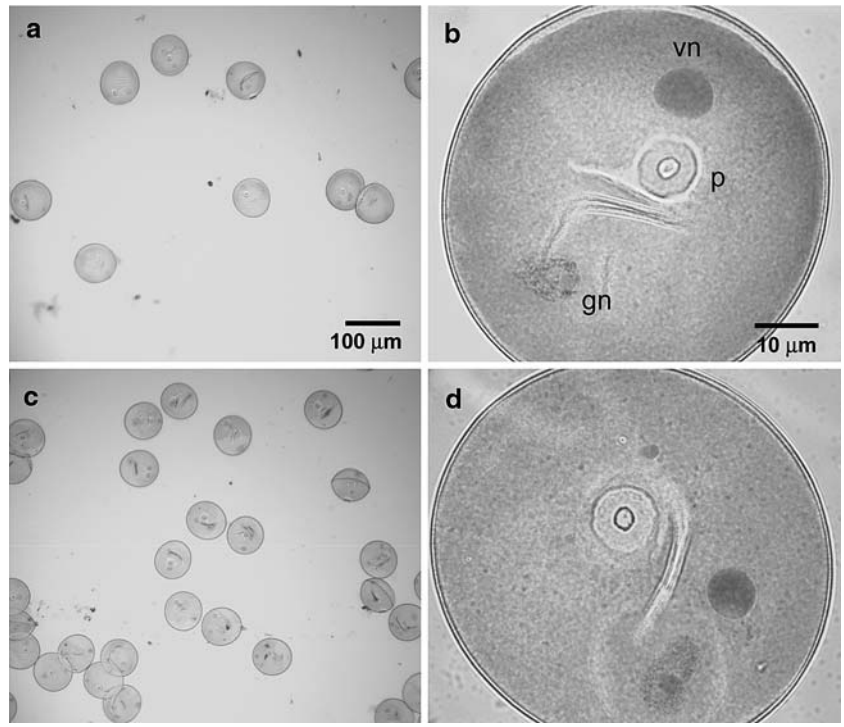

Fig. 6 Comparison of mitotic stage microspores from single anther of upper florets of P2P31-182 (**a**, **b**) and P2P31-190 (**c**, **d**) stained with propiocarmine. p, pore; vn, vegetative nucleus; gn, generative nucleus

Table 4 Herbicide screening to frequency of *bar* gene transmission through pollen

Event	Total	Herbicide screen		PCR positive[a]	Ratio alive/total	Ratio PCR positive/total
		Dead	Alive			
P2P31-144	144	135	9	17	0.063	0.118
P2P31-182	139	136	3	3	0.022	0.022
P2P31-190	147	147	0	0	0.000	0.000
P2P31-097	146	146	0	1	0.000	0.007
P2P46-7	145	124	21	64	0.169	0.441

[a] All plants were analyzed by PCR for the presence of both bar and Glu1Dx5 and these transgenes co-segregated in every case

experiment, plants that successfully transmitted and expressed the transgene locus through pollen should survive the herbicide treatment, while those that do not express the transgene locus should die. Because it is possible that the transgene locus could be transferred through pollen but silenced, we used PCR to determine if the transgene locus was present in the plants prior to applying the herbicide.

The frequency of pollen transmission from transgenic plants was expressed as the frequency of plants carrying the transgene locus, so normal pollen transmission from transgenic plants would result in a frequency of 50% resistant plants. Limited pollen transmission was observed in P2P31 events 144, 182, and 97 at rates of 11.8 %, 2.2% and 0.7%, respectively, and no transmission through pollen was observed in P2P31 event 190. In contrast, the P2P46 normal pollen transmission control plants had a pollen transmission frequency of 44.1%, somewhat less than the 50% expectation, but higher than that of the P2P31 plants. The herbicide resistance gene was clearly transmitted at low frequency through pollen, presumably because of its linkage to the wheat genomic fragment. This demonstrates the feasibility of using the wheat genomic fragment to control pollen transmission of a transgene.

Discussion

A segment of wheat DNA causes poor pollen transmission of transgenes in maize

A wheat genomic fragment containing the *Glu-1Dx5* gene that was not transmitted efficiently through pollen has been reported (Sangtong et al. 2002). All four independent transformation events that were characterized exhibited similarly low (0–10%) pollen transmission efficiencies, while plants created with the same protocol but with different transgene constructs (for example, P2P46 described here, as well as other constructs (Yang et al. 2002)) did not. This suggested that the wheat genomic DNA fragment was somehow responsible for the low rate of pollen transmission of transgene loci. The wheat genomic fragment contained in the P2P31 construct is about 9 kb long, with the 1Dx5 coding sequence occupying approximately the middle third of this fragment (Fig. 1). The portion of the fragment 5′ of the *Glu-1Dx5* promoter contained 15 open reading frames, ranging in length from 52 to 166 codons.

To determine if the 5′ portion of the wheat genomic DNA fragment was responsible for the reduced pollen transmission, we made transgenic plants bearing a construct designated P2P46 in which this region was replaced with the maize 27 kDa γ-zein promoter. Plants carrying P2P46 exhibited normal pollen transmission as indicated in Table 1, and normal pollen transmission in two out of the three crosses indicated in Table 2. One P2P46 cross in Table 2 gave a 1:1 ratio when a heterozygous plant was self pollinated. A likely explanation for this ratio is gene silencing. We saw evidence of this in a subsequent experiment (Table 4). Thus, in contrast to P2P31, we did not find evidence of poor pollen transmission in P2P46 events. The number of transformation events we have characterized is relatively small (four for P2P31 and four for P2P46), and it is possible that the pollen transmission is event specific, rather than a property of the construct. This possibility is unlikely given the relatively low frequency of pollen transmission problems in transgenic plants. The only difference between the two constructs was that the γ-zein promoter replaced the wheat genomic DNA fragment 5′ of the 1Dx5 coding sequence. Thus, we concluded that this region of the wheat genomic DNA fragment was responsible for reduced pollen transmission in these plants.

Though the tassels and anthers of plants containing P2P31 appeared morphologically normal, it was possible that reduced pollen transmission of P2P31 was caused by the transgene interfering with pollen development as a gametophytic mutant. In this case, no transgenic pollen would be shed. Alternatively, pollen carrying the transgene could have been shed, but had reduced viability. Our results supported the latter possibility. Those events with abnormal transmission of the transgene displayed pollen with varying degrees of morphological abnormalities. In the extreme case, one pollen class was normal in size and viability and the other class was small and essentially non-viable. The extent of the morphological abnormalities observed does not correlate completely with the degree of pollen transmission reported in Table 2. This may be because the degree of unusual pollen morphology is event specific and not directly related to poor pollen transmission. Abnormal pollen morphology associated with non-viability or male sterility in mutants or transgenic material (McCormick 2004, Mariani et al. 1990, Muschietti et al. 1994, Pline et al. 2002, Sari-Gorla et al. 1997) and with chromosomal aberrations including deficiencies and duplications, translocations, and inversions (Burnham 1950, Stadler and Roman 1948) has been reported. Our observations of binucleate microspores were consistent with a model in which all pollen undergoes normal development up to the binucleate microspore stage. At an unknown stage thereafter, development of pollen carrying the transgene is perturbed. In this model, wheat genomic DNA fragment contained in the P2P31 transgene functions as a gametophytic mutant that interferes with the later stages of development of transgenic pollen. The degree of morphological difference that this causes in the pollen varies, and may be event specific.

We have localized the region of wheat DNA responsible for controlling pollen transmission of maize transgene loci to a 3899 bp fragment and we have shown that this fragment was necessary to cause abnormal pollen development. We have not

established that this DNA fragment is sufficient to cause abnormal pollen development nor have we identified a specific mechanism of action. It is known that certain chromosomal abnormalities interfere with pollen transmission, so it is possible that the wheat DNA fragment induced chromosomal abnormalities that interfered with pollen development. We consider this to be an unlikely possibility because it would require the wheat genomic DNA to induce a chromosomal abnormality that did not affect gene expression of the transgene locus but was severe enough to interfere with pollen development. A more likely explanation is that the transgene encodes a factor that interferes with pollen development when expressed in maize. This factor could be the 1Dx5 protein, however, this protein was not detectable in pollen (Sangtong et al. 2002). Furthermore, the results associated with the P2P46 construct showed that 1Dx5 had little effect on pollen development when expressed using the maize 27 kDa γ-zein promoter. Another possibility is that the wheat genomic DNA fragment carried a factor encoded by one of the open reading frames and this factor interfered with pollen development.

Potential for use for controlling pollen transmission

These data suggest a system for controlling pollen transmission of maize transgenes. Transgenes of interest could be co-transformed with the wheat DNA fragment used in this study so that the two transgenes are genetically linked. Transgenic grain could then be produced using plants heterozygous for the transgene locus, and pollen transmission of the transgene locus would be reduced by the presence of the wheat genomic fragment. Transgene-induced male gametophytic mutations that interfere with pollen development, pollen tube germination or fertilization could be used in place of the wheat genomic fragment. For example, a transgenic down-regulation of the *LAT52* protein that resulted in pollen incapable of fertilization (Muschietti et al. 1994) would be a good candidate. The advantages of a system based on a transgene-induced gametophytic mutant are that: (1) it theoretically can be used with any transgene and would be implemented at the time of transformation; (2) there is no requirement to cross transgene loci into a specific genetic background for this system to work, as would be the case in some proposed systems (Evans and Kermicle 2001; Stevens et al. 2004); and (3) it would not be necessary to plant a pollinator with the transgenic plants in order to produce grain, because the transgenic plants shed viable (although non-transgenic) pollen. A disadvantage of this system is that transgenic plants would have to be propagated as heterozygotes, so only half of the grain produced would be transgenic. It would also be necessary to select transgenic seed to plant each season.

All systems proposed for control of transgene loci have a possibility of ''leaks''. In one of the four events we studied, we did not detect transmission through the pollen. In the other three, however, we detected pollen transmission at a rate of less than 10%. Since the rate of pollen transmission varied among events, it should be possible to minimize pollen transmission by selecting events with the lowest degree of pollen transmission. While it is not possible to guarantee absolute control of pollen transmission of transgenes, the possibility of effectively controlling pollen transmission would be increased by using several systems in combination.

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